EXHIBIT 3

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Production of Human Compatible High Mannose-type ($Man_5GlcNAc_2$) Sugar Chains in *Saccharomyces cerevisiae**

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A yeast mutant capable of producing $Man_5GlcNAc_2$ human compatible sugar chains on glycoproteins was constructed. An expression vector for α-1,2-mannosidase with the "HDEL" endoplasmic reticulum retention/retrieval tag was designed and expressed in *Saccharomyces cerevisiae*. An *in vitro* α-1,2-mannosidase assay and Western blot analysis showed that it was successfully localized in the endoplasmic reticulum. A triple mutant yeast lacking three glycosyltransferase activities was then transformed with an α-1,2-mannosidase expression vector. The oligosaccharide structures of carboxypeptidase Y as well as cell surface glycoproteins were analyzed, and the recombinant yeast was shown to produce a series of high mannose-type sugar chains including $Man_5GlcNAc_2$. This is the first report of a recombinant *S. cerevisiae* able to produce $Man_5GlcNAc_2$-oligosaccharides, the intermediate for hybrid-type and complex-type sugar chains.

Saccharomyces cerevisiae is useful for the production of recombinant proteins of biological interest because of the established expression system, and it can be easily grown in large quantities. Moreover, yeast share the early steps of the mammalian Asn-linked glycosylation pathway. However, the mature Asn-linked oligosaccharides of yeast are mannan glycans and are highly antigenic against mammals. Thus, it would be necessary to eliminate the antigenicity of the sugar chains when recombinant therapeutic glycoproteins are produced in yeast.

Several genes concerned with the biosynthesis of yeast sugar chains have been cloned, and the glycosylation pathway of yeast has been clarified. The *OCH1* gene encodes an α-1,6-mannosyltransferase that initiates α-1,6-polymannose outer chain formation on the Asn-linked inner oligosaccharide $Man_8GlcNAc_2$ in *S. cerevisiae* (1). *MNN1* has been proposed as the structural gene for the α-1,3-mannosyltransferase that elongates the outer chain and the inner core oligosaccharide (2, 3). The Δ*och1 mnn1* double mutant accumulated a single oligosaccharide moiety, $Man_8GlcNAc_2$, a high mannose-type structure (1). This mutant may be useful to produce recombinant therapeutic glycoproteins without any antigenicity toward humans.

On the other hand, some glycoproteins of therapeutic value require complex-type sugar chains for their efficacy. Erythropoietin (EPO),[1] a hematopoietic glycoprotein factor produced in the kidney, has three complex-type Asn-linked sugar chains and one mucin-type sugar chain. It is reported that the composition and structure of each sugar chain affected the biological activity, the efficiency of secretion, and had profound effects on the half-life of EPO in the blood circulation (4). It seems that the most active form of the EPO molecule requires tetraantennary Asn-linked sugar chains (5) with full sialylation, to prevent serum clearance by the action of the hepatic asialoglycoprotein binding protein (6, 7). When EPO was expressed in the Δ*och1 mnn1* mutant yeast, the recombinant EPO should have high mannose-type oligosaccharides, which are trapped by the mannan-binding proteins of serum, liver, and macrophages, or excreted in the urine through the kidney because of their small size.

From the viewpoint of glycotechnology, we are trying to construct the mammalian-type glycosylation system in *S. cerevisiae* as a host to produce glycoprotein therapeutics (Fig. 1). The first aim of this research was to convert the mannan-type sugar chain of *S. cerevisiae* to a $Man_5GlcNAc_2$ sugar chain, because it is an intermediate for hybrid- and complex-type sugar chains. However the Δ*och1 mnn1* mutant can only produce the $Man_8GlcNAc_2$ structure (1). Further trimming of the mannose residues by α-1,2-mannosidase requires α-mannosidase I. Several α-1,2-mannosidases have been isolated from mammals, yeast, and fungi (8), and some mammalian α-1,2-mannosidase genes have been cloned (9, 10). During preparation of the manuscript, it was reported that a truncated soluble form of the human α-1,2-mannosidase IB was expressed as a secreted protein in *Pichia pastris* (11). The *S. cerevisiae* α-1,2-mannosidase gene (*MNS1*) has been cloned (12) and expressed in *S. cerevisiae*. However, this enzyme only removes a specific single mannose residue from $Man_9GlcNAc_2$ and produces $Man_8GlcNAc_2$. The *Aspergillus* α-1,2-mannosidase gene (*msdS*) has also been cloned and has been expressed successfully in yeast cells as a chimeric gene with the signal sequence of the aspergillopepsin I gene from *Aspergillus saitoi* (13, 14). The recombinant α-1,2-mannosidase activity was secreted into

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[1] The abbreviations used are: EPO, erythropoietin; ER, endoplasmic reticulum; CPY, carboxypeptidase Y; endo-H, endo-β-*N*-acetylhexosaminidase; PA, 2-aminopyridine; PMSF, phenylmethylsulfonyl fluoride; GnT−, *N*-acetylglucosaminyltransferase; SD, synthetic minimal dextrose; CL, crude lysate; LSP, low speed pellet; HSP, high speed pellet; HSS, supernatant fraction; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; MES, 2-morpholinoethansulfonic acid.

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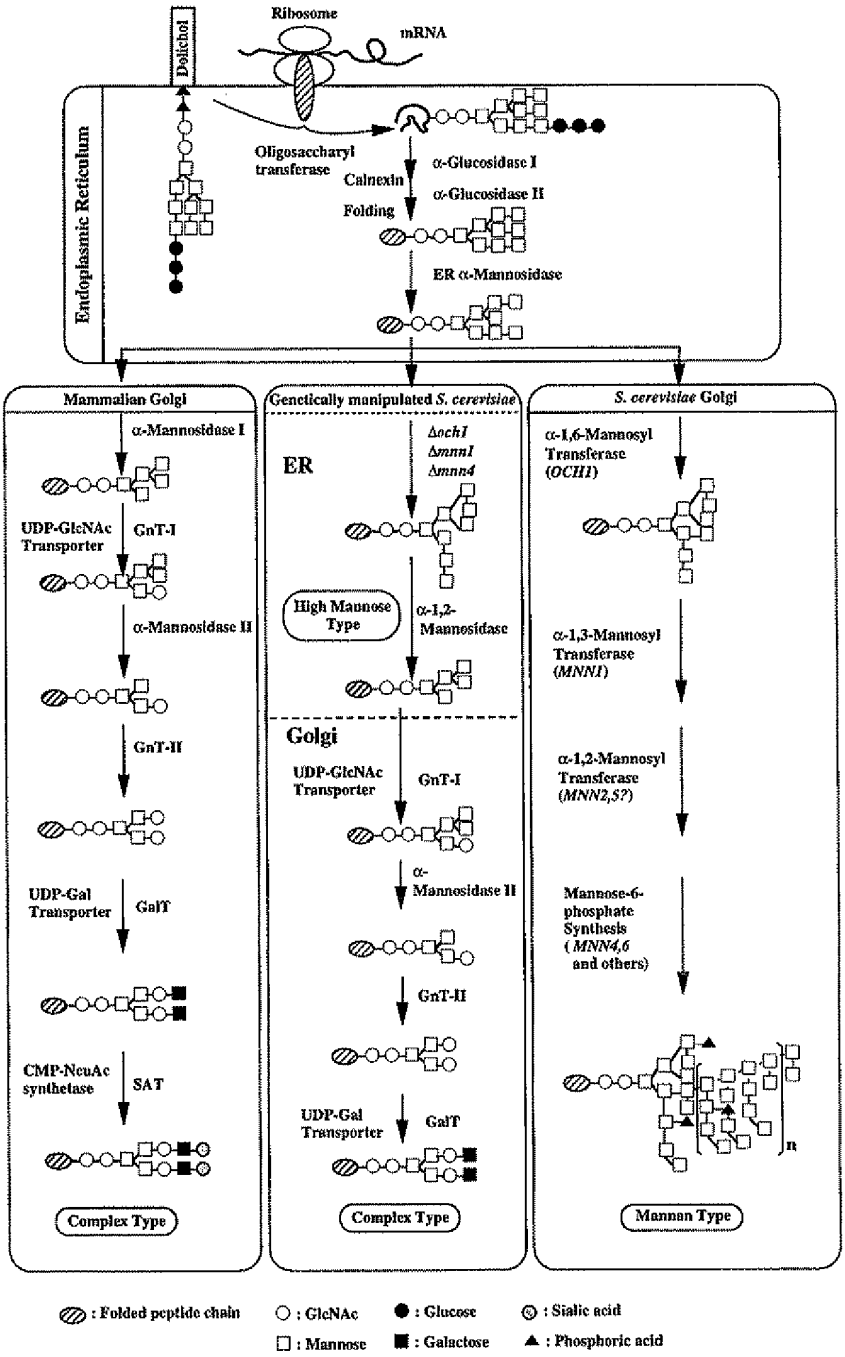

FIG. 1. **Strategy for genetic manipulation of *S. cerevisiae*, and the comparison of the *N*-glycosylation pathway in mammalian cells and *S. cerevisiae*.**

the culture medium, indicating that the products of the *msdS* gene had passed through the yeast secretion pathway. Therefore, α-1,2-mannosidase could be used as a tool to produce the mammalian-type sugar chains in the yeast if this enzyme was retained in the endoplasmic reticulum (ER) or Golgi apparatus.

In yeast cells, the His-Asp-Glu-Leu (HDEL) C-terminal sequence of proteins acts as a retention/retrieval signal for the endoplasmic reticulum (ER) (15). Proteins with an HDEL sequence are bound by a membrane-bound receptor (Erd2p) (16, 17) and then enter a retrograde transport pathway for return to the ER from the Golgi apparatus. In this study, the expression of the *A. saitoi* α-1,2-mannosidase in the ER was demonstrated by adding "HDEL" to the C terminus of the α-1,2-mannosidase open reading frame. The introduced α-1,2-mannosidase was also shown to convert Asn-linked oligosaccharides into $Man_5GlcNAc_2$, the intermediate form for hybrid- and complex-type sugar chains, in mutant yeast cells with disruptions in three of the original mannosyltranferase genes (*OCH1*, *MNN1*, and *MNN4*).

EXPERIMENTAL PROCEDURES

Yeast Strain and Culture Conditions—The enzyme activity and the localization of the HDEL-tagged MsdSp was determined in *S. cerevisiae pep4* disrupted YPH500 cells (*MATα ura3–52 lys2–801 ade2–101 trp1-Δ63 his3-Δ200 leu2-Δ1 pep4::ADE2*) (18). YS132–8B (*MATα och1::LEU2 mnn1::URA3 mnn4::LYS2 leu2-Δ1 ura3–52 trp1-Δ1 lys2–801^{AM} his3-Δ200 ade2–101^{OC}*), which had been constructed by standard genetic methods (19), were used to analyze the Asn-linked sugar chains of carboxypeptidase Y (CPY) or mannoproteins. All strains were transformed by the method of Ito *et al.* (20). Transformants were selected on synthetic minimal dextrose (SD) medium with auxotrophic supplements.

DNA Constructs—For preparation of the HDEL-tagged MsdSp, the tag sequence was introduced by amplifying the 0.6-kilobase region between the *Hin*dIII site and the stop codon of the *msdS* gene with the following mutation primers: 5′-TGCGCCCGGAAGTGATTGAA-3′ and 5′-CCTACAATTCGTCGTGTGTACTACTCACCCGCACTGG-3′. The

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polymerase chain reaction product was subcloned into pCR-Script Amp SK(+) (Stratagene) and digested with *Hin*dIII and *Not*I. The coding region of the C-terminal domain of pGAM1, an expression plasmid for *A. saitoi* α-1,2-mannosidase (13), was substituted with the recovered 0.6-kilobase *Hin*dIII-*Not*I fragment to create the pGAMH1 plasmid. The *msdS* and insertion sequence were confirmed by DNA sequencing.

α-1,2-Mannosidase Assay—$Man_6GlcNAc_2$ oligosaccharide was obtained from Seikagaku Co. (Tokyo, Japan) and labeled with 2-aminopyridine (21). Pyridylaminated oligosaccharide ($Man_6GlcNAc_2$-PA) was purified by gel filtration (TOYOPEARL HW-40, 1.6 × 73 cm, Tosoh Corp., Japan) and the purity confirmed by reversed-phase HPLC using an ODS-80T_M column (0.46 × 15 cm, Tosoh Corp.).

Yeast cell extracts were prepared as described below. Yeast cells were cultured on SD medium lacking tryptophan. The cell density was determined at 600 nm using a 10-mm cuvette. The pelleted cells were washed with deionized water, resuspended in extraction buffer (0.1 M sodium acetate buffer (pH 5.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% Triton X-100), and vortex mixed with acid-washed glass beads (425–600 μm diameter). Soluble cell extract was separated from cell debris by centrifugation and assayed for activity. Samples containing 10–100 μg of protein were incubated for 30 min with 150 pmol of $Man_6GlcNAc_2$-PA in 0.1 M sodium acetate buffer (pH 5.0) at 37 °C. The assay was stopped by boiling, and the sample was filtrated using an Ultrafree-MC centrifugal filter unit (0.22 μm pore size low-binding Durapore, Millipore). The filtrates were analyzed by HPLC with a Hitachi F-1050 fluorescence spectrophotometer, using an ODS-80T_M column (4.6 × 150 mm). The solvent and elution conditions used are as described by Kondo *et al.* (21). One unit of the enzyme was defined as the amount of enzyme that was required to liberate 1 μmol of mannose from $Man_6GlcNAc_2$-PA per min at 30 °C and pH 5.0.

Marker Enzyme Assay—NADH cytochrome P-450 reductase, a marker enzyme for the ER, guanosine diphosphatase, a marker enzyme for the Golgi apparatus, and glucose-6-phosphate dehydrogenase, a cytosol marker, were assayed as described (22–24), respectively.

Western Blot Analysis—Rabbit anti-MsdSp and rabbit anti-glucose-6-phosphate dehydrogenase antisera were obtained from Sawaday Technology (Tokyo, Japan). Mouse anti-CPY monoclonal antibody 10A5-B5, mouse anti-alkaline phosphatase monoclonal antibody 1D3-A10, and mouse anti-dolichol phosphate mannose synthase monoclonal antibody 5C5-A7 were purchased from Molecular Probes, Inc. (Eugene, OR).

Samples containing 1 μg of protein were subjected to SDS-PAGE. SDS-PAGE was carried out using the buffer system of Laemmli (25) in 10% gel. Electroblotting of the fractionated proteins onto polyvinylidene difluoride membrane (Millipore Corp.) was carried out by the method of Towbin *et al.* (26), and detection was performed essentially according to the method of Hsu *et al.* (27).

Subcellular Fractionation—Cells were grown in SD medium and were converted to spheroplasts by the method of Vita *et al.* (28). The following procedures were also performed at 4 °C. The spheroplasts were harvested by centrifugation. The spheroplasts were resuspended in a hypoosmotic lysis buffer (0.25 M sorbitol, 10 mM triethanolamine (pH 7.2), 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, antipain (2 μg/ml), chymostatin (2 μg/ml), pepstatin A (3 μg/ml), leupeptin (2 μg/ml)) and homogenized for up to 20 times using a glass tissue homogenizer. The lysate was centrifuged at 220 × *g* for 5 min to remove unlysed spheroplasts. The 220 × *g* supernatant (CL) was centrifuged at 10,000 × *g* for 15 min to separate the low speed pellet (LSP) and supernatant fractions. The supernatant was centrifuged at 100,000 × *g* for 80 min to separate the high speed pellet (HSP) and supernatant fractions (HSS). The LSP and HSP were resuspended by sonication on ice in lysis buffer containing 1% Triton X-100. Aliquots of the LSP, HSP and HSS fractions were used to assay α-1,2-mannosidase, NADH cytochrome P-450 reductase, guanosine diphosphatase, and glucose-6-phosphate dehydrogenase activities and were also subjected to Western blot analyses.

Aliquots (200 μl) of the resuspended LSP fraction were placed on top of four 1.8-ml 1.2/1.5 M discontinuous sucrose gradients containing 10 mM triethanolamine (pH 7.2). After centrifugation at 80,000 × *g* in an RT-100T Beckman Ultracentrifuge at 4 °C for 65 min, 200 μl each were collected from the top of each gradient and pooled. Aliquots of the pools were resuspended as described above and were subjected to Western blot analysis.

Endo-β-N-acetylglucosaminidase H Treatment—Recombinant endo-H was purchased from Genzyme Co. (Boston, MA). Yeast cell extracts were prepared as described above. Aliquots (containing 15 μg of proteins) of cell extracts were brought to 50 μl of 50 mM sodium citrate buffer (pH 6.0) containing 0.1% SDS and 1 mM PMSF and denatured at 100 °C for 5 min. After dilution with 50 mM sodium citrate buffer (pH 6.0) containing 1 mM PMSF, 0.5 milliunits of endo-H was added to the sample and incubated for 16 h at 37 °C. Samples that substituted buffer for endo-H were used as negative controls. The samples containing 1 μg of protein were subjected to SDS-PAGE and analyzed by Western blotting with mouse anti-CPY antibody.

Purification of CPY—*p*-Aminobenzylsuccinic acid was purchased from Sigma. CNBr-activated Sepharose 4B was obtained from Amersham Pharmacia Biotech, and glycyl-tyrosine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). CPY produced in YS132–8B cells harboring pG3 or pGAMH was purified using affinity column chromatography. The affinity gel was prepared by coupling the specific inhibitor, *p*-aminobenzylsuccinic acid, via an azo linkage to Sepharose-glycyl-tyrosine as described by Johansen *et al.* (29). Before use, the column (1.0 × 3.0 cm) was equilibrated with 10 mM MES buffer (pH 5.0). *S. cerevisiae* YS132–8B and transformed YS132–8B cells were cultivated in SD medium containing 0.3 M sorbitol at 30 °C and harvested at stationary phase. The culture was centrifuged at 220 × *g* for 5 min, and the pellet was disrupted as described above. The yeast cell lysate was applied to the column and washed extensively with 500 ml of 1 M NaCl in 10 mM sodium acetate buffer (pH 4.3). Elution was performed with 10 mM phosphate buffer (pH 7.0). The eluate was concentrated by Centricon-10 (Nihon Millipore Ltd., Japan) and applied to SDS-PAGE. The purified CPY was lyophilized and subjected to Asn-linked oligosaccharide analysis.

Preparation of Mannoprotein—*S. cerevisiae* YS132–8B and transformed YS132–8B cells were cultivated in SD medium containing 0.3 M sorbitol at 30 °C and harvested at mid-log phase. Mannoproteins were extracted by hot citrate buffer (0.1 M citrate buffer, pH 7.0) followed by precipitation with ethanol (30). The precipitates were further purified by a Concanavalin A-agarose column (0.8 × 2 cm, Honen Corp., Japan), which was equilibrated with Con A buffer (0.1 M Tris-HCl (pH 7.2) containing 0.15 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$). The column was eluted by the Con A buffer containing 0.2 M α-methyl-D-mannoside. The eluted fractions were dialyzed against water and lyophilized.

HPLC Analysis of Asn-linked Oligosaccharides on CPY and Mannoproteins—*N*-glycanase was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). CPY or mannoproteins were dissolved in 100 mM sodium phosphate buffer (pH 7.2) containing 0.5% SDS and 50 mM 2-mercaptoethanol and then denatured at 100 °C for 5 min. After dilution with 100 mM sodium phosphate buffer and addition of 0.5% Nonidet P-40, 2.5 units of *N*-glycanase was added to the sample and incubated for 16 h at 37 °C. Liberated Asn-linked oligosaccharides were separated from the salts and peptides using an AG 501-X8 mixed bed resin (Bio-Rad), and from Nonidet P-40 using Bio-Beads S-X8 (Bio-Rad). Reductive pyridylamination and structural analyses of the purified oligosaccharides were carried out essentially according to the method of Kondo *et al.* (21). Pyridylaminated (PA-) oligosaccharides were analyzed by HPLC using a size-fractionation column (TSKgel Amide-80, 4.6 × 250 mm, Tosoh Corp.) and a reversed-phase column (TSKgel ODS-80T_M, 4.6 × 150 mm). Authentic PA-oligosaccharides and PA-glucose oligomer were purchased from Takara Shuzo Co. (Kyoto, Japan).

RESULTS

Expression of the α-1,2-Mannosidase—Measurement of α-1,2-mannosidase activity by the Nelson-Somogyi method was attempted but was not sensitive enough to detect the amount of enzyme present. We have developed a new assay for α-1,2-mannosidase using a fluorescent oligosaccharide. PA-oligosaccharide made it possible to assay picomole per minute-ordered enzyme activity. For the assay of the α-1,2-mannosidase activity *in vitro*, we used $Man_6GlcNAc_2$-PA oligosaccharide as a substrate. The optimal assay conditions, such as enzyme concentration, reaction time, and substrate concentration were determined as described under "Experimental Procedures."

A soluble form of *A. saitoi* α-1,2-mannosidase was constructed with the HDEL ER retention/retrieval signal sequence at the C terminus. This construct was subcloned into the multicopy plasmid pG3, termed pGAMH1, and was used to transform *S. cerevisiae* YPH500 cells. α-1,2-Mannosidase activity that converted $Man_6GlcNAc_2$-PA substrate into $Man_5GlcNAc_2$-PA was observed in the cell extracts of the recombinant yeast with the pGAMH1 vector (Fig. 2*B*), whereas there was no such activity in the extract of the recombinant yeast transfected

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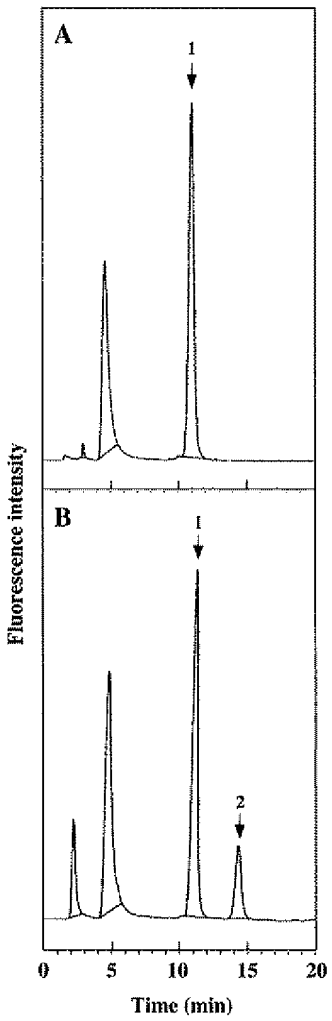

FIG. 2. ***In vitro* α-1,2-mannosidase assay of cell extracts of the recombinant yeasts.** The cell extract was incubated with 10 μM pyridylaminated 6-oligomannose type sugar chain ($Man_6GlcNAc_2$-PA) at 30 °C in a final volume of 15 μl for 30 min. After incubation, the α-1,2-mannosidase activity was measured by HPLC analysis. Chromatograms of the reaction product of the yeast cell extract harboring null vector (*panel A*) and harboring α-1,2-mannosidase-HDEL expression plasmid (*panel B*) were displayed. *Peaks 1*, $Man_6GlcNAc_2$-PA; *peak 2*, $Man_5GlcNAc_2$-PA.

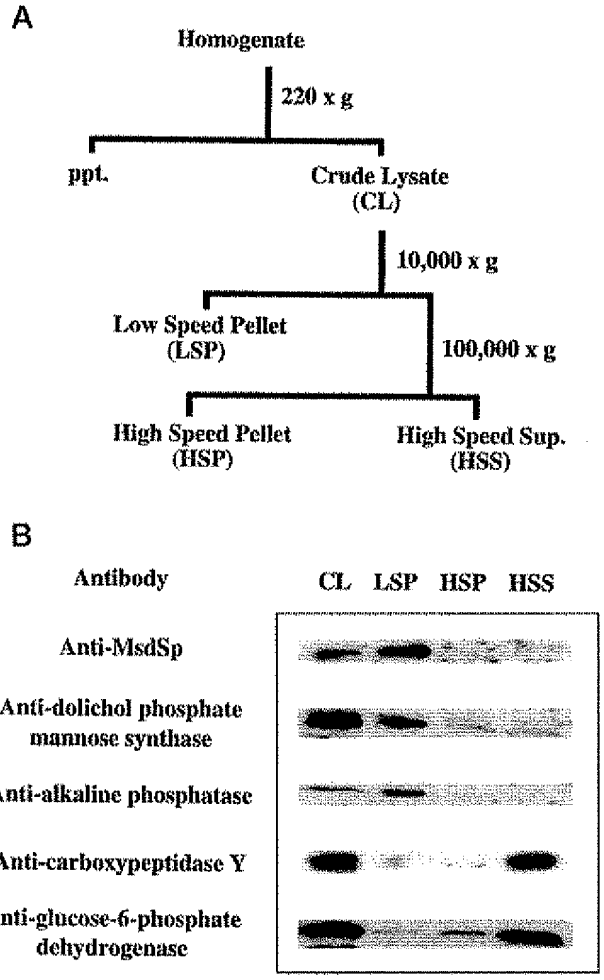

FIG. 3. **Subcellular fractionation of the recombinant α-1,2-mannosidase.** Wild-type (YPH500) spheroplasts harboring pGAMH1 were osmotically lysed. *A*, lysed spheroplasts were subjected to differential centrifugation as described under "Experimental Procedures." CL, LSP, HSP, and HSS fractions were subjected to Western blotting and enzyme assays. *ppt*, precipitate. *B*, Western blot analysis was carried out using various yeast protein antibodies.

TABLE I
Subcellular distribution of the recombinant α-1,2-mannosidase

Enzyme[a]	Localization	Distribution			Recovery[b]
		LSP	HSP	HSS	
		%			
α-1,2-Mannosidase		77	10	13	79.5
Cytochrome P-450 reductase	ER	69	14	17	90.5
Guanosine diphosphatase	Golgi	18	70	12	124.8
Kex2p	Late Golgi	8	52	40	123.1
Glycerol-6-phosphate dehydrogenase	Cytosol	7	17	76	83.4

[a] Each enzyme activity was assayed as described under "Experimental Procedures."
[b] Recovery present in 220 × *g* supernatant (CL) was taken as 100 %.

with the pG3 vector only (Fig. 2*A*). The activity of ER α-1,2-mannosidase and vacuole α-mannosidase in yeast were not detected under these assay conditions. Four milliunits of the enzyme activity was recovered from a 500-ml yeast culture.

Localization of the α-1,2-Mannosidase—To determine the localization of the expressed α-1,2-mannosidase in yeast, we investigated the subcellular distribution of the enzyme. Fig. 3*A* illustrates the protocol for the fractionation of the yeast cells.

The α-1,2-mannosidase activity was localized primarily in the LSP fraction (77%) (Table I). The LSP fraction also contained 69% of NADPH cytochrome P-450 reductase (ER marker) (31, 32). In contrast, most of the guanosine diphosphatase (70%), a Golgi marker (23), was found in the HSP fraction. Kex2p (33), a late Golgi marker, was split into the HSP and HSS fractions. The cytosol marker, glucose-6-phosphate dehydrogenase, was detected mainly in the HSS fraction (76%).

The Western blot pattern also showed that the ER marker protein (dolichol phosphate mannose synthase) and the vacuolar membrane protein (alkaline phosphatase) were localized in the LSP fraction, whereas both CPY, which is a soluble protein in the vacuole, and cytosolic glucose-6-phosphate dehydrogenase were fractionated in HSS fraction (Fig. 3*B*). The introduced α-1,2-mannosidase gene products were detected in the LSP fraction. Because it is known that the LSP fraction contained the vacuole in addition to the ER (34), discontinuous sucrose density centrifugation was performed to determine whether the expressed α-1,2-mannosidase was localized in the ER or the vacuole (Fig. 4). Alkaline phosphatase, a vacuolar marker enzyme, was distributed to fractions 1–3, the most light density fraction. In contrast, dolichol phosphate mannose synthase, an ER marker, was distributed to fractions 4–6. This result indi-

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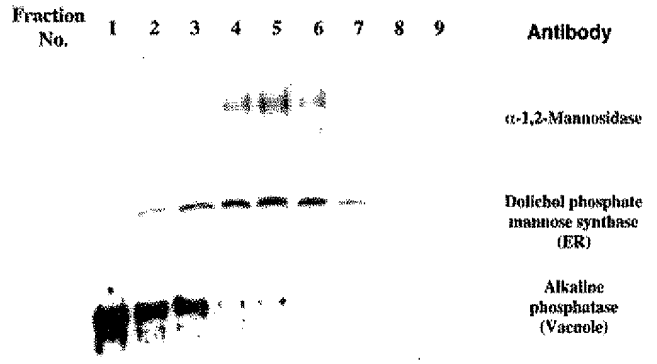

FIG. 4. **Distribution of α-1,2-mannosidase and marker proteins after discontinuous sucrose density centrifugation.** Sucrose gradient was fractionated into nine fractions. *Fraction 1* came from the top (the lightest fraction) to the bottom (the heaviest fraction).

cated that the vacuole and ER are well separated from each other in this system. The signals of the α-1,2-mannosidase appeared around fraction 5. The results strongly suggested that the α-1,2-mannosidase with the HDEL-tag is mainly localized in the ER.

Oligosaccharide Structures of the Recombinant Triple Mutant Yeast—The apparent molecular mass of the CPY produced in the recombinant yeasts was analyzed on SDS-PAGE followed by Western blot analysis. YS132–8B, which has disrupted *OCH1*, *MNN1*, and *MNN4* genes, will not have any outer mannosyl chains on its glycoproteins. As shown in Fig. 5, the CPY from YS132–8B carrying the null vector gave a single signal with an apparent molecular mass of 62 kDa on SDS-PAGE. However the CPY from YS132–8B harboring the pGAMH1 plasmid gave an additional signal below the original one, indicating that the sugar chains of the CPY have been trimmed by the introduced α-1,2-mannosidase. Treatment of each cell lysate with endo-H gave a single signal of an *N*-deglycosylated CPY (Fig. 5, *third* and *fourth lanes*).

The oligosaccharide structures of glycoproteins produced in these yeasts were analyzed using CPY as a model glycoprotein. While the sugar chains of CPY produced in the yeast with the null vector were eluted at the $Man_8GlcNAc_2$-PA position on the amide column (Fig. 6A, *graph a*), those produced in the yeast with the pGAMH1 plasmid showed four peaks at positions corresponding from $Man_5GlcNAc_2$-PA to $Man_8GlcNAc_2$-PA, respectively (Fig. 6A, *graph b*). The molar ratio of each glycoform was $Man_5GlcNAc_2$-PA:$Man_6GlcNAc_2$-PA:$Man_7GlcNAc_2$-PA:$Man_8GlcNAc_2$-PA = 27:22:22:29. The fraction eluted at the position corresponding to $Man_5GlcNAc_2$-PA (indicated with a *open arrow* in Fig. 6A) was pooled and subjected to reversed-phase chromatography. Only one peak was observed at the same position as authentic Manα1–3[Manα1–3(Manα1–6)Manα1–6]Manβ1–4GlcNAcβ1–4GlcNAc-PA (Fig. 6*B*); this is the smallest structure of mammalian-type high-mannose sugar chains.

Besides CPY, we also investigated the oligosaccharide structures of cell wall mannoproteins. As shown in Fig. 6*C*, the mannoproteins produced in the yeast with the pGAMH1 plasmid contained $Man_5GlcNAc_2$. The molar ratio of each glycoform in mannoproteins was $Man_5GlcNAc_2$-PA:$Man_6GlcNAc_2$-PA:$Man_7GlcNAc_2$-PA:$Man_8GlcNAc_2$-PA = 10:13:16:61.

DISCUSSION

α-Mannosidase I digests α-1,2-mannosidic linkages and converts $Man_8GlcNAc_2$ oligosaccharide into $Man_5GlcNAc_2$. This is the first step in the biosynthesis of hybrid-type and complex-type sugar chains from high mannose-type sugar chains. There are several successive enzymatic reactions necessary to com-

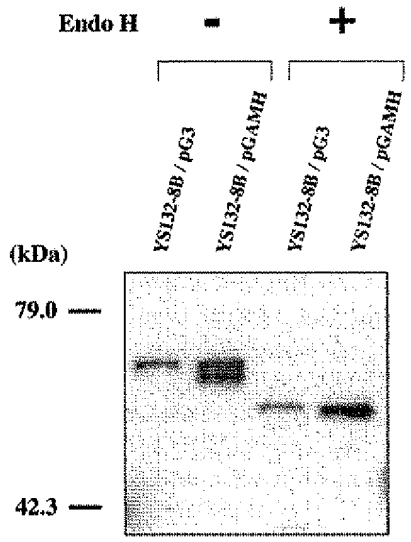

FIG. 5. **Western blot analysis of the carboxypeptidase Y from the recombinant yeast cell extracts.** *S. cerevisiae* YS132–8B strain (Δ*och1* Δ*mnn1* Δ*mnn4*, triple mutant) was used as a host. *First* and *third lanes*, from YS132–8B harboring the null vector; *second* and *fourth lanes*, from YS132–8B harboring the expression plasmid, pGAMH1. Endo-H digestion resulted in the shift of the signals corresponding to the deglycosylated form (*third* and *fourth lanes*).

plete complex-type structures. *N*-Acetylglucosaminyltransferase (GnT)-I, α-mannosidase II, GnT-II, β-1,4-galactosyltransferase, etc. work in succession in mammalian cells. Since the α-1,2-mannosidase acts upstream in the biosynthetic pathway of oligosaccharides, it must be located either in the ER or the early Golgi apparatus to reconstruct this system in yeast. We have already succeeded in expressing the *A. saitoi* α-1,2-mannosidase as a chimeric protein with a transmembrane domain of Och1p (data not shown). Although Och1p resides in the early Golgi apparatus of yeast (35), the expressed chimeric enzyme was localized not only in the Golgi apparatus, but also in the ER and the cytosol fractions. We could not detect any $Man_5GlcNAc_2$ sugar chain structure in the recombinant yeast (data not shown). Evidence suggested that the mislocalization of the chimeric α-1,2-mannosidase prevented the trimming of sugar chains in the yeast. In this study, we attempted to localize the α-1,2-mannosidase to the yeast ER using a retention/retrieval signal.

There has been several retention/retrieval systems proposed to date. Some of these systems require a transmembrane domain and/or a cytoplasmic tail. Xaa-Xaa-Arg-Arg (*XX*RR; *X* is any amino acid) in the N-terminal cytoplasmic region and Lys-Lys-Xaa-Xaa (KK*XX*), in the C-terminal cytoplasmic domain of membrane proteins are known as retrieval signals for the ER. It has also been demonstrated that the N-terminal 16 amino acids of the alkaline phosphatase in the cytoplasmic tail contain a vacuolar sorting signal in *S. cerevisiae* (36). Lussier *et al.* reported that an N-terminal cytoplasmic domain was necessary for Kre2p to correctly localize in the Golgi apparatus and that the entire Kre2p cytoplasmic tail plus the transmembrane domain and 36 amino acids in the luminal stem region were required to localize a Pho8p reporter protein in the yeast Golgi apparatus (35). These results suggested that there is no accurate signal for the retention of exogenous membrane proteins in the ER or Golgi apparatus of yeast. Therefore, we constructed an expression vector with an HDEL signal for the transfer of soluble α-1,2-mannosidase proteins from the Golgi apparatus to the ER.

In yeast, two α-mannosidases have been found, and these have different substrate specificity and pH optima to the *A.*

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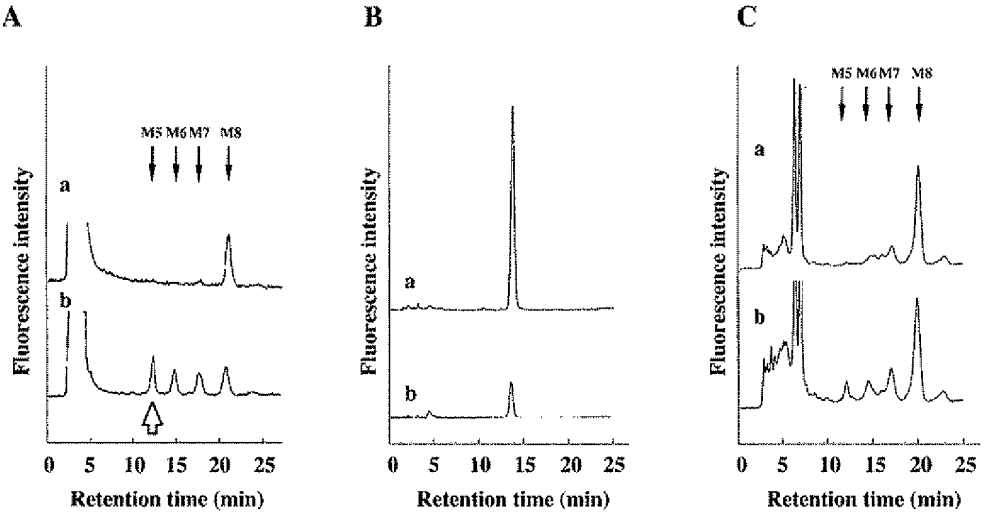

FIG. 6. **Analysis of Asn-linked oligosaccharides in the triple mutant strain YS132–8B.** *A*, chromatogram of the sugar chains of CPY on HPLC using a TSKgel Amide-80 column. *Graph a*, from YS132–8B harboring null vector; *graph b*, from YS132–8B harboring the expression plasmid, pGAMH1. *B*, the peak indicated by the *open arrow* in *panel A* was pooled and subjected to HPLC using a TSKgel ODS-80T$_M$ column. *Graph a*, standard sugar chain of Manα1–3[Manα1–3(Manα1–6)Manα1–6]Manβ1–4GlcNAcβ1–4GlcNAc-PA. *Graph b*, the pooled fraction in *panel A*. *C*, chromatogram of the sugar chains of mannoproteins on HPLC using a TSKgel Amide-80 column. *Graph a*, from YS132–8B harboring null vector; *graph b*, from YS132–8B harboring the expression plasmid, pGAMH1. The elution times of authentic PA-sugar chains were indicated by *arrows*. M5, $Man_5GlcNAc_2$-PA; M6, $Man_6GlcNAc_2$-PA; M7, $Man_7GlcNAc_2$-PA; M8, $Man_8GlcNAc_2$-PA.

saitoi α-1,2-mannosidase. *S. cerevisiae* ER α-mannosidase (Mns1p) cannot act on $Man_6GlcNAc_2$ oligosaccharide, and vacuolar α-mannosidase (Ams1p) cannot act at pH 5.0. Whereas *A. saitoi* α-1,2-mannosidase can remove the α-1,2-linked mannose of $Man_6GlcNAc_2$ oligosaccharide at pH 5.0. Based on these facts, we have developed an assay method that specifically detects *A. saitoi* α-1,2-mannosidase activity.

The subcellular fractionation experiments indicated that the product of the *msdS* gene was mainly localized in the LSP fraction (Fig. 3), which includes the ER, vacuole, and plasma membrane. However, it is unlikely that MsdSp was localized in the vacuole, because the signal distribution of MsdSp was quite different from that of CPY, which is the vacuolar marker (Fig. 3). Furthermore, MsdSp will never be anchored at the plasma membrane because it is a soluble protein. The fractionation in the sucrose discontinuous gradients also showed that the signal distribution of the product of the *msdS* gene did not match with that of the vacuole but with that of the ER.

CPY was chosen as one of the reporter glycoproteins to analyze the glycosylation phenotype of the genetically constructed yeast, because it has four Asn-linked oligosaccharides of known structure (37), and it has an established purification method (29). The triple mutant strain (YS132–8B) used in this study lacks three of the yeast mannosyltransferase activities, and the elongation of *N*-glycan is terminated at the $Man_8GlcNAc_2$ structure (1), which is a substrate for the α-1,2-mannosidase. Structural analysis of the CPY sugar chains produced in the mutant yeast harboring the pGAMH1 plasmid showed that the introduced α-1,2-mannosidase digested the sugar chains up to $Man_5GlcNAc_2$ (Fig. 6). The fact that the mannoproteins of the yeast with pGAMH1 vector also had the $Man_5GlcNAc_2$ structure suggests that the introduced α-1,2-mannosidase could digest the oligosaccharide chains of secretary proteins. The observed lower molar ratio of $Man_5GlcNAc_2$ in mannoproteins might be because of the cell harvesting period; the mannoproteins were recovered at mid-log phase of the culture, whereas CPY was done at stationary phase. Because there were also intermediates ranging from $Man_8GlcNAc_2$ to $Man_6GlcNAc_2$ both on CPY and mannoproteins, the expression level of the introduced α-1,2-mannosidase seemed not to be sufficient for complete trimming of each sugar chain. It might be more suitable to produce therapeutic glycoproteins using a vector with an inducible promoter, such as *CUP1* or *GAL1* promoter. We could induce production of a target protein after stationary phase, where α-1,2-mannosidase would be sufficiently expressed to convert all of the sugar chains to $Man_5GlcNAc_2$.

In this study, *S. cerevisiae* was manipulated to produce $Man_5GlcNAc_2$ *N*-glycan. Increasing the efficiency of the α-1,2-mannosidase reaction remains to be done. Furthermore, the $Man_5GlcNAc_2$ *N*-glycan is a hybrid and a complex-type intermediate, the latter of which is better suited and more effective for human therapeutics. We have already succeeded in expressing GnT-I, GnT-II, and β-1,4-galactosyltransferase activities in yeast, but to make hybrid- and complex-type sugar chains in yeast cells, co-expression of GnT-I and α-1,2-mannosidase is required and is an object of our future research.

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